DOI: 10.2478/s11686-007-0048-6

Acta Parasitologica, 2007, 52(4), 299–304; ISSN 1230-2821

VERSITA

Sarcocystis arctosi sp. nov. (Apicomplexa, Sarcocystidae) from the brown bear (*Ursus arctos*), and its genetic similarity to schizonts of *Sarcocystis canis*-like parasite associated with fatal hepatitis in polar bears (*Ursus maritimus*)

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Abstract

The tissues of herbivores are commonly infected with cysts of parasites belonging to the apicomplexan genus *Sarcocystis,* but such sarcocysts are rare in bears. Here, we describe a new species, *Sarcocystis arctosi*, based on the mature sarcocysts identified in two brown bears (*Ursus arctos*) from Alaska, USA. Microscopic sarcocysts (37–75 × 20–42 µm) had thin walls (<1 µm). The outer layer of the sarcocyst, the parasitophorous vacuolar membrane (pvm), was wavy in outline and had minute undulations that did not invaginate towards the sarcocyst interior; these undulations occurred at irregular intervals and measured up to 100 nm in length and up to 60 nm width. The ground substance layer beneath the pvm was smooth and lacked microtubules. Longitudinally cut bradyzoites measured 5.6–6.8 × 0.7–1.8 µm. A major portion of nuclear small subunit rDNA sequence obtained from these sarcocysts was similar to that previously obtained from the hepatic schizonts of a *S. canis*-like parasite from polar bears (*Ursus maritimus*).

Keywords

Protozoa, Apicomplexa, *Sarcocystis arctosi* sp. nov., brown bear, *Ursus arctos*

Introduction

Parasites belonging to the apicomplexan genus *Sarcocystis* depend upon predation to effect their transmission (Dubey *et al*. 1989). Prey and predators serve, respectively, as intermediate and definitive hosts for these parasites. The definitive host becomes infected by ingesting the asexual stage (sarcocyst) encysted in the intermediate host's tissues, whereupon the sexual cycle may commence in the lamina propria of the small intestine of the carnivore. Typically, species of *Sarcocystis* exclusively parasitize a single intermediate host species.

Encysted parasites commonly occur in the tissues of herbivores, but are rare in bears. Previously, sarcocysts of unnamed species were found in sections of six of 53 black bears (*Ursus americanus*) from the southeastern United States (Crum *et al*. 1978), one of 92 in North Carolina (Dubey *et al*. 1998), one of 132 from Florida (Cheadle *et al*. 2002), and two of 46 in Oregon (Foreyt *et al*. 1999).

In the present study we report mature sarcocysts from two brown bears (*Ursus arctos*) in Alaska, USA, for the first time from this host.

Materials and methods

Heads from two brown bears killed in defense of human life on the Kenai Peninsula, Alaska [bear no.1, Alaska Department of Fish and Game (ADFG) no. 0603433, sub-adult male killed on October 10, 2006 in Clam Gulch, Alaska 60°2´N, 151°4´W and bear no. 2, an adult female ADFG l no. 0603434, killed on October 11, 2006 in Soldotna, Alaska 60°5´N, 151°4´W] were shipped to the Animal Parasitic Diseases Laboratory (APDL), United States Department of Agriculture, Beltsville for parasite evaluation. Approximately 50 g of tongue from both bears were digested in acid pepsin solution and the digest was examined microscopically for protozoa (Dubey *et al*. 1989).

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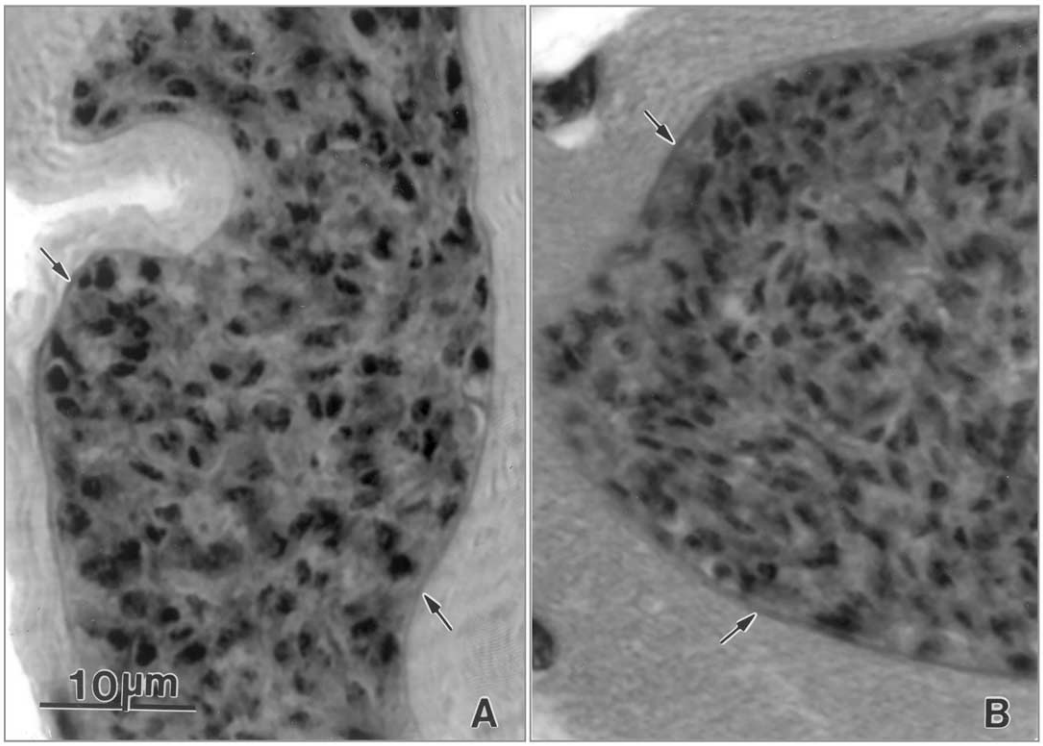

Fig. 1. Sarcocysts of *Sarcocystis arctosi* sp. nov. in skeletal muscles of the naturally-infected brown bear. Note thin sarcocyst wall (arrows)

Tongue and muscles from the neck area from each bear was fed to two cats, one for each bear, and the faeces of cats were examined for coccidian oocysts (Dubey 1995). Four weeks later, the cats were killed and homogenates of their small intestine were digested in 5.25% sodium hypochlorite solution (Chlorox) and the digest was examined microscopically for *Sarcocystis* sporocysts (Dubey *et al*. 1989).

Samples of tongue and muscles from throat and neck were fixed in 10% buffered neutral formalin on October 17, 2006, one week after the discovery of bradyzoites in digest of tongue. Routine histologic examination was performed on paraffin-embedded sections (5 µm) stained with haematoxylin and eosin (H and E).

For transmission electron microscopy, paraffin-embedded tongue sections were post-fixed in 1% osmium tetroxide in Millonig's phosphate buffer, rinsed in the same buffer, dehydrated in ethanol and embedded in epoxy resin. Semithin sections were stained with toluidine blue in 1% sodium tetraborate. The ultrathin sections were contrasted with uranyl acetate and lead citrate before examination in a transmission electron microscope.

For molecular characterization, the centrifuged sediment from each tongue digest was stored at –70°C and further digested for DNA extraction, using the DNeasy tissue kit (Qiagen Corp.) for animal tissues (these procedures were also performed on two additional vials to which no tissue was added in order to confirm the absence of contaminating DNA in our reagents or equipment). Each specimen was then subjected to PCR amplification of a 1484 bp portion of 18S rRNA gene and directly sequenced on an ABI 3100 instrument after removing excess primers and unincorporated nucleotides using ExoSAP-IT (USB Corp.). Sequences were edited using Sequencher (GeneCodes Corp.) and compared to all available homologues identified by BLAST in the nonredundant nucleotide GenBank database. Multiple sequence alignments of all such sequences were constructed using CLUSTALW, and phylogenetic relationships inferred under the criterion of minimum evolution using Kimura 2-parameter distances using MEGA version 3.1 (Kumar *et al*. 2004).

Results

Sarcocystis-like bradyzoites were seen in the pepsin digest of tongue of both bears. These bradyzoites were slender and estimated to be 5–7 µm long but were not measured. A total of five microscopic sarcocysts were seen from both sarcocysts in numerous sections of tongue and other muscles. In bear no. 1, only one sarcocyst was seen and it measured 62.5 × 27.5 µm in a section of skeletal muscle. Four sarcocysts were seen in bear no. 2 (1 in tongue, and 3 in muscles, including 2 in one myocyte); these sarcocysts measured 75 × 42.5 µm, 75 × 37.5 µm, 37.5 × 30 µm, and 37.5 × 20 µm. In 5 µm sections stained with H and E, the sarcocyst wall appeared thin (<1 µm) and smooth (Fig. 1A, B). Septa were present. The sarcocyst interior was packed with slender bradyzoites that were

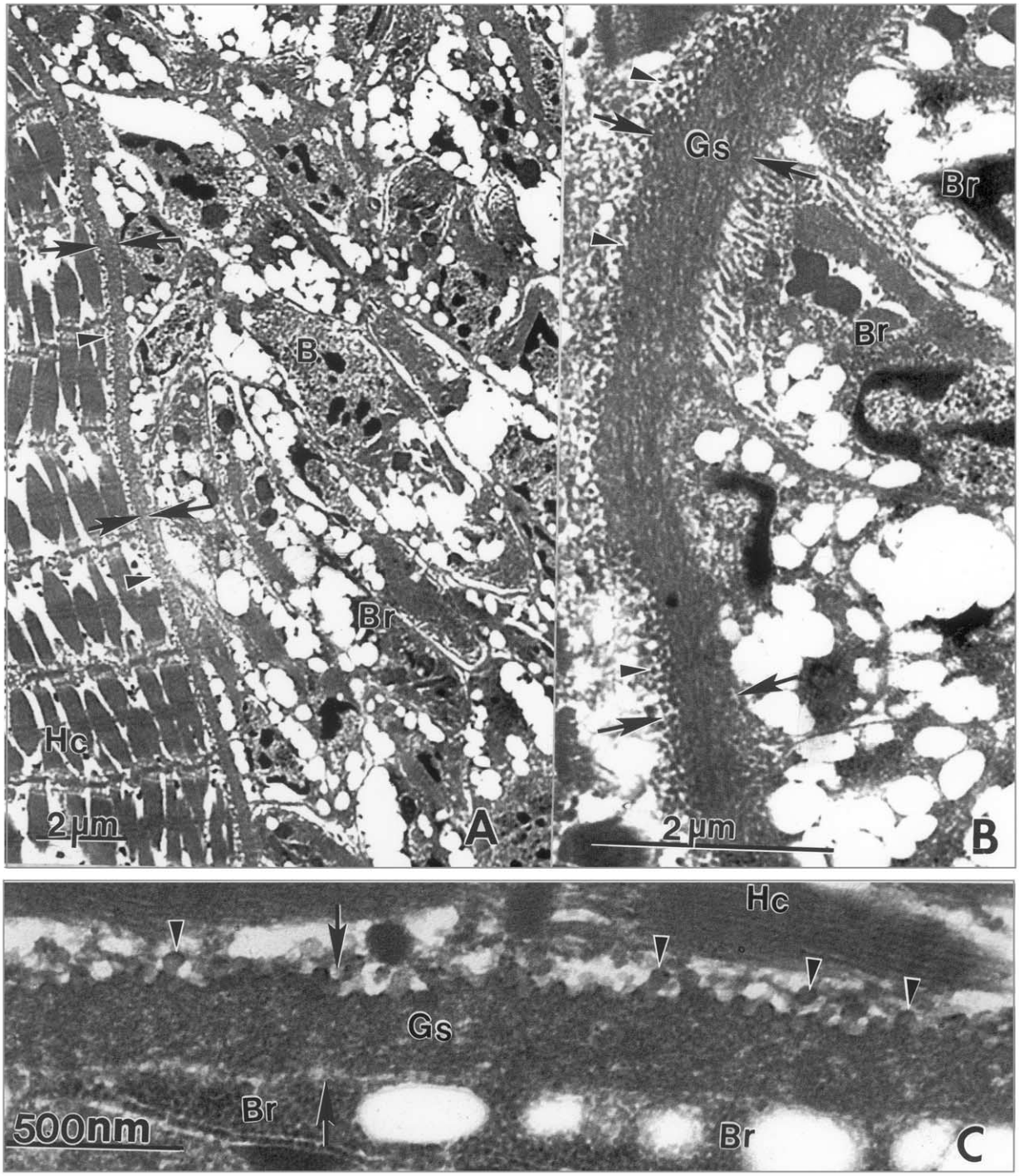

Fig. 2. TEM of the sarcocyst of *Sarcocystis arctosi* sp. nov. cut at different angles (**A**, **B**, **C**) within a host cell (Hc). Opposing arrows indicate the sarcocyst wall. Arrowheads point to minute undulations on the sarcocyst wall. The bradyzoites (Br) are located just beneath the smooth ground substance (Gs) layer

difficult to measure because their boundaries were indistinct. Out of all five paraffin blocks processed for thin sections, only one sarcocyst (from bear no. 2) was seen in 1-µm sections stained with toluidine blue; villar protrusions were not visible on the sarcocyst wall of this sarcocyst.

Sections of one sarcocyst were examined ultrastructurally (Fig. 2). The outer layer of the sarcocyst, the parasitophorous vacuolar membrane (pvm), was wavy in outline and had minute undulations that did not invaginate towards the sarcocyst interior (Fig. 2). These undulations occurred at

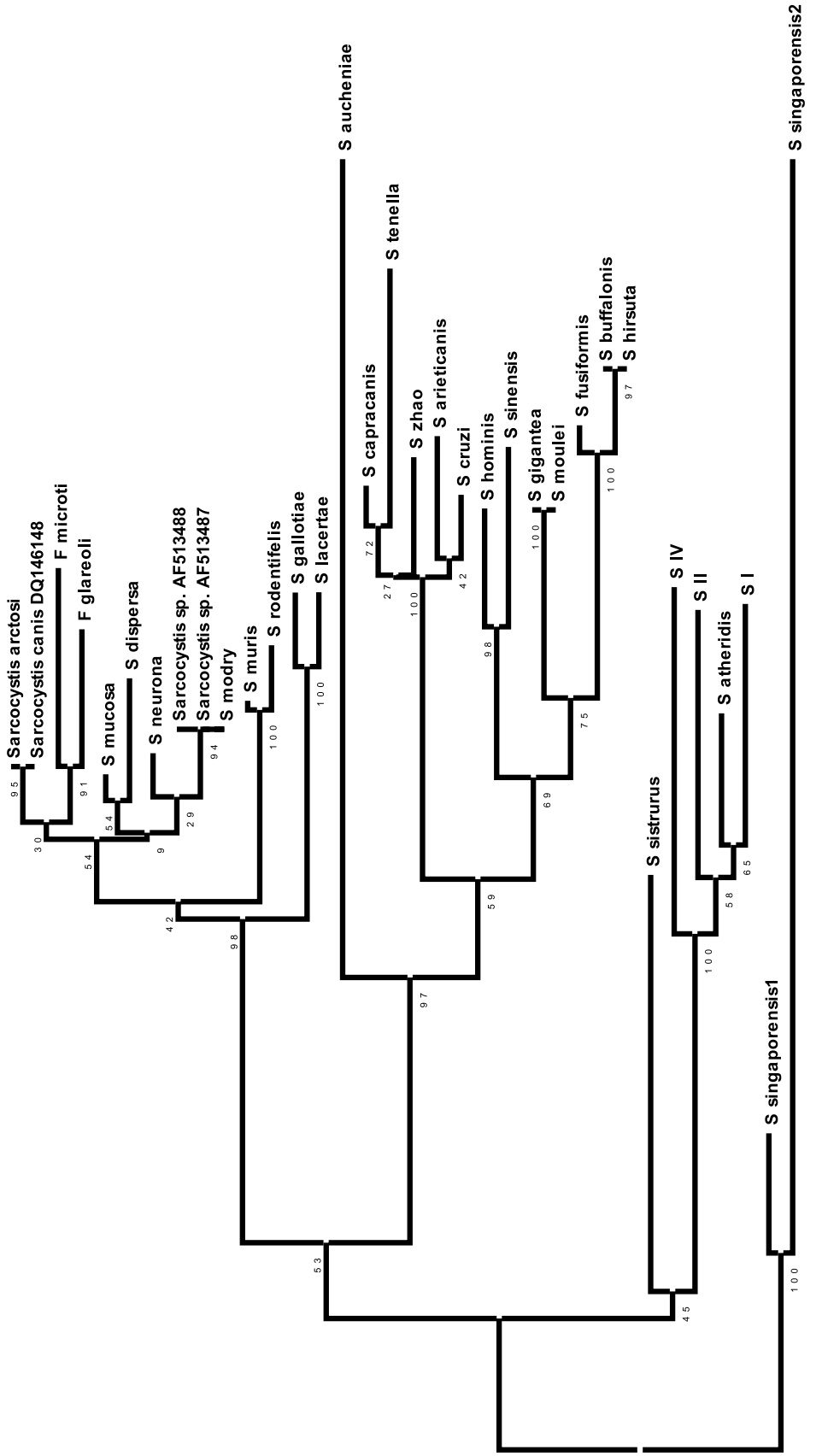

Fig. 3. Phylogenetic relationships of *Sarcocystis arctosi* sp. nov. (GenBank accession no. EF564590) to *Sarcocystis canis* and other related parasites reconstructed from variation in 18S rDNA under the criterion of minimum evolution and using Kimura 2-parameter pairwise distances. Node support indicated from 500 bootstrap replicates of the data

irregular intervals and measured up to 100 nm in length and up to 60 nm in width. The interior of the undulations was electron-dense. The ground substance (Gs) layer beneath the pvm was smooth and lacked microtubules. The Gs was approximately 0.4–0.5 µm wide and of uniform thickness except when cut at an angle (Fig. 2). The sarcocyst was mature and contained fully formed bradyzoites; metrocytes were absent. Groups of bradyzoites were separated by septa. Longitudinally cut bradyzoites measured 5.6–6.8 × 0.7–1.8 µm (n = 5). Bradyzoites contained a conoid, micronemes, 1–2 rhoptries per section, and a posteriorly located nucleus. The rhoptries had a long neck and were often looped so that the blunt end was directed towards the conoid. Micronemes were few and located throughout the bradyzoite. The nucleus was located in the posterior half of the parasite. Amylopectin granules were present in the posterior three quarters of the bradyzoites.

A partial sequence representing most of the small subunit rDNA (1484 bp) was sequenced (GenBank no. EF564590) and used as the basis of a BLAST search of the non-redundant nucleotide database and was compared with related taxa (Fig. 3). Surprisingly, this sequence was identical, over the entire 993 bp available for comparison, to a sequence previously derived from schizonts in the liver of a polar bear attributed to *S. canis (*Dubey *et al*. 2006).

No *Sarcocystis* sporocysts were observed in either the faeces or the intestinal scrapings of either cats fed *Sarcocystis*-infected bear tissues.

Discussion

Complete life-cycles of *Sarcocystis* are known for only a few species of animals, mostly those employing livestock intermediate hosts (Dubey *et al*. 1989). Most *Sarcocystis* species have been named based on their intermediate host occurrence and their structure. Among all diagnostic morphological criteria, the structure of the sarcocyst wall is most valuable for differentiating those species that share a given host. Dubey *et al*. (1989) and Dubey and Odening (2001) recognized 35 types of sarcocyst walls based on their structure.

The wall of the sarcocyst described here from the brown bear is distinct. It most closely resembles Type 1 sarcocyst wall because of minute undulations on the wall. However, it differs from Type 1 because it does not invaginate in to the interior of the sarcocyst (Dubey *et al*. 1989). It also appears distinct from the unnamed species of *Sarcocystis* previously reported from black bears (Dubey *et al*. 1998), whose sarcocyst wall had 2-µm long villar protrusions.

Interestingly, the sequenced portion of rDNA of the sarcocysts in brown bear could not be distinguished from the schizonts previously reported from a *S. canis*-like parasite previously reported from the liver of a polar bear that died in a zoo at Anchorage, Alaska (Garner *et al*. 1997). Fortunately, a sample of liver from one of these polar bears, kept frozen for 10 years, was subsequently successfully characterized genetically (Dubey *et al*. 2006). The rDNA from schizonts in the polar bear was found to be distinct from any other species of *Sarcocystis* then known (Dubey *et al*. 2006).

Sarcocystis canis is a poorly described parasite; only the schizont stage is known. *Sarcocystis canis* was named based on only the schizont stage to draw attention to a disease causing fatal hepatitis in dogs (Dubey and Speer 1991). Subsequently *S. canis*-associated hepatitis was diagnosed in additional dogs, a sea lion, a chinchilla, a dolphin, a Hawaiian monk seal, a horse, a black bear (reviewed in Dubey *et al*. 2006). In all of these cases, only schizonts were seen, the infection was confined to the liver, and no material was available for parasite cultivation.

The precise agreement of the rDNA of the previously reported *S. canis* from that polar bear with the present study from brown bears raises the interesting possibility that they may represent, respectively, the schizont and sarcocyst stages of the same etiological agent. Although subsequent analysis may yet discriminate among these two putative taxa, their especially strong resemblance in any event establishes that they share an especially close evolutionary relationship. Whether or not recognizable distinctions are ultimately identified between them, this serendipitous finding of phylogenetic affinity may provide an important clue to unraveling the mystery of enigmatic-but-widespread occurrences of fatal parasitic hepatitis, by providing a detailed prediction of the sarcocysts whose consumption induces such disease.

To aid in efforts to elucidate parasite epidemiology and biodiversity, and to facilitate precision in the functional application of taxonomic nomenclature during a period of admitted uncertainty, we have chosen to designate sarcocysts from brown bear parasite as *S. arctosi*. In the event that complete characterization of the life-cycle of *S. canis* establishes it as indistinguishable on all relevant grounds, the name *S. canis* should enjoy priority.

Taxonomic summary

Diagnosis: Sarcocysts microscopic with <1 µm thick sarcocyst walls. Minute undulations on the sarcocyst wall present that do not invaginate in to the interior of the sarcocyst.

Type host: Brown bear (*Ursus arctos*).

Other hosts: Unknown.

Type locality: Alaska, USA.

Etymology: Derived from the host genus.

Specimens deposited: Two histological sections stained with H and E from both bears (D5391 and D5390), and one section from bear D5391 were deposited in the United States National Parasite Collection (USNPC nos.100080 and 100081), United States Department of Agriculture Beltsville, Maryland 20705, USA.

Acknowledgements. We would like to thank Larry Lewis and Jeff Selinger of the Alaska Department of Fish and Game for their help in obtaining bear samples, Detiger Dunams for expert PCR and sequencing, and John Jenkins, Armed Forces Institute of Pathology, Washington, D.C. for electron microscopy.

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(Accepted August 10, 2007)